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Nobiletin represses change in the levels of blood coagulation markers in the LPS-induced rat DIC model

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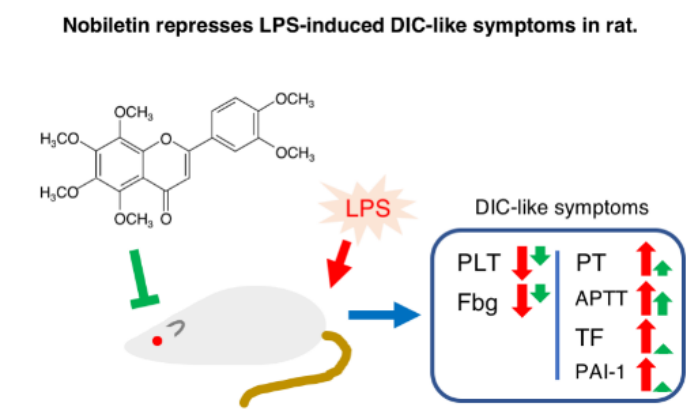
ABSTRACT

Background: Nobiletin is contained in Shiikuwasa fruit, a popular citrus fruit from Okinawa Prefecture in Japan. Nobiletin reportedly acts as a strong antioxidant, an anti-inflammatory agent, and an anti-cancer agent, and it suppresses the expression of TF which triggers blood coagulation. However, *in vivo* verification of *in vitro* reports is necessary. This study used a rat model of LPS-induced microthrombosis based on the *in vivo* studies as previously reported. Sustained intravenous injection of LPS changed all blood coagulation indicators in the direction of thrombus formation. The aim of this study was to determine if intake of nobiletin could suppress DIC-like symptoms.



Methods: Experimental SD rats were fully anesthetized and fixed to an operating table. Either LPS alone or nobiletin (50 mg/kg) plus LPS was given to rats to investigate the repressive effects of nobiletin on the expression of blood coagulation factors.

Results: After 4 h of LPS infusion (12.5 mg/kg/h, i.v.), PLT counts and Fbg levels in rat plasma decreased by 80% and 74%, respectively. PT and APTT were extended by 180% and 256%, respectively. TF activity and PAI-1 antigen levels were remarkably increased (54- and 86-fold, respectively vs. control). Pretreatment on nobiletin (50 mg/kg, p.o.) reduced or suppressed fluctuations in blood coagulation indices caused by LPS. TF activity was repressed almost completely by nobiletin pretreatment. After 4 h, PAI-1 antigen levels in nobiletin-treated animals were repressed 82.6% compared to LPS-treated rats. Nobiletin repressed LPS-induced changes in TF and PAI-1 more effectively than other parameters. Further, nobiletin repressed fibrin thrombi formation in the renal glomeruli induced by LPS treatment.

Conclusions: Nobiletin was found to reduce LPS-induced DIC-like symptoms in rats. In the fluctuations of blood indices related to the coagulation cascade, nobiletin suppressed the LPS-induced expression of PAI-1 and TF more effectively than other indices. The binding sites of transcription factors that are activated by LPS-induced signals reside in the promoter areas of TF and PAI-1 gene sequences. Thus, the suppression of TF and PAI-1 expression by nobiletin appears similar to mechanisms previously evaluated during *in vitro* experiments. Importantly, nobiletin repressed fibrin deposition in the renal glomeruli induced by LPS treatment and improved overall health. Nobiletin may function as an anti-thrombogenic agent when ingested daily.

Keywords: nobiletin; LPS; DIC model; blood coagulation; anti-thrombogenic

BACKGROUND

Disseminated intravascular coagulation (DIC) is characterized by systemic activation of the hemostatic system and it is associated with high mortality in patients with sepsis [1-2]. Excessive coagulation activation, inhibition of fibrinolysis and consumption of coagulation inhibitors lead to a hypercoagulable state, resulting in fibrin deposition in microvessels and inflammatory reactions [3]. In septic shock, it is thought that several inflammatory cytokines liberated from endothelial cells and monocytes or lipopolysaccharide (LPS, endotoxin) released from Gram-negative bacteria play a major role in the formation of such pathological conditions [4].

A common *in vivo* model of DIC is based on the infusion of thromboplastin or LPS into rats [5-6]. However, there are differences in the formation of DIC-like pathology between the two models. The fibrinolytic system predominates in thromboplastin-induced DIC [7] whereas the coagulation system is predominant in the LPS-induced DIC model [8]. In addition, in the thromboplastin model, coagulation factors directly cause blood coagulation, whereas in the LPS model, blood coagulation occurs after expression of coagulation adhesion factors. In the LPS model, the

expressions of these coagulation factors are controlled with a signal transduction system in vascular endothelial cells and monocytes after LPS binds to TLR4 on CD14+ cells [8-9].

Nobiletin (5,6,7,8,3′, 4′-hexamethoxyflavone) is a polymethoxy flavonoid contained in the rind of citrus fruits (Figure 1).

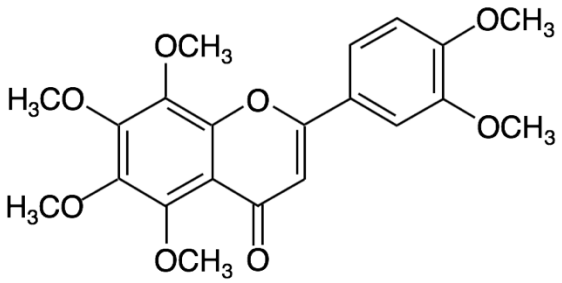


Figure 1. The structure of nobiletin

It exhibits pharmacological effects, including anti-inflammatory activity [10], as well as inhibition of tumor proliferation, invasion, and metastasis [11-12]. In addition, we previously reported that nobiletin was expected to possess anti-coagulant effects through repression of tissue factor (TF) [13] and thrombin-activatable fibrinolysis inhibitor (TAFI) expression [14]. However, all of these findings were obtained from *in vitro* studies using cultured monocyte-like cells and hepatocytes, and the actual effects *in vivo* were unknown. Recently, it was reported that nobiletin prevented arterial thrombosis in mice using a fluorescein-induced platelet thrombi model [15]. We were interested in whether nobiletin actually suppresses pathological blood coagulation reactions *in vivo*.

Previously, our studies showed that nobiletin repressed the expression of blood coagulation factors through transcriptional inhibition [13-14]. Consequently, we anticipated that nobiletin might be a useful blood coagulation inhibitor when consumed daily in food [15]. Therefore, we tested that hypothesis in rats by using the LPS-induced coagulation model because it involves the activation of transcriptional factors and coagulation factors. Here we report that nobiletin suppressed the changes of thrombotic indices in the blood of LPS-treated DIC model rats.

METHODS

Chemicals and Reagents: Nobiletin was a gift from Dr. A. Ito [16]. Other reagents were purchased from Wako Pure Chemical Industries Co. (Osaka, /Japan) unless otherwise indicated.

Model of microthrombosis induced by LPS followed by nobiletin administration: Rat models of LPS-induced microthrombosis were generated as reported previously [5-6]. Six- to seven-week-old male Sprague-Dawley rats with body weights ranging from 200 to 250 g were obtained from Sankyo Laboratory Service (Tokyo, Japan). Experiments were conducted in 5 animals per a group. Rats were anesthetized by an intraperitoneal injection of pentobarbital (50 mg/kg). Microthrombi were induced by infusion of 12.5 mg/kg/h LPS (*Escherichia coli* 0111:B4, Sigma, L2630) via the femoral vein for 30 min. Nobiletin was administered orally 30 min before LPS infusion. The rats were sacrificed 4 h after the beginning of LPS infusion, and blood samples were collected. Experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, 1996), and were performed after receiving approval from the ethics committee of Showa Pharmaceutical University.

Measurement of hematological parameters: Blood samples were collected into tubes containing 1/10 volume of 3.8% sodium citrate for measurement of platelet (PLT) count, fibrinogen (Fbg) concentration, prothrombin time (PT), activated partial

thromboplastin time (APTT), plasminogen activator inhibitor-1 (PAI-1) antigen and TF activity, or into special tubes containing thrombin for measurement of fibrin degradation products (FDP). PLTs in the blood were counted with a Coulter Counter model ZM (Beckman Coulter, Inc., Brea, CA). Fbg concentration, PT and APTT in plasma were determined by a coagulation time assay using the Amelung KC-4A Micro (MC medical, Tokyo, Japan). Fbg concentrations were measured with TriniCLOT ® Fibrinogen Kit (Kyowa Medex Co., Ltd. Tokyo, Japan). PPP was diluted with imidazol buffer in the kit (1:10) and 60 µL of diluted PPP was warmed at 37℃ for 2 min in the cuvette. Then, 30 µL of thrombin test solution was added. The time of coagulation was determined automatically by the blood coagulation measuring system Amelung KC-4A Micro (MC medical, Tokyo, Japan). PT was measured with Nicoplastin® (rabbit brain thromboplastin with calcium, Sanko Jun-yaku, Tokyo, Japan). PPP (100 µL) was warmed at 37℃ for 1 min in the cuvette, and then 200 µL of Nicoplastin solution was added. Coagulation time was measured by KC-4A. APTT was measured with APTT test solution in the Thrombo Check APTT® (Sysmex, Tokyo, Japan). APTT test solution (100 µL) was warmed at 37℃ for 1 min in the cuvette, then 100 µL of PPP was added and warmed at 37℃ for 2 min. The coagulation time after the addition of 0.1 mL of 0.02 M calcium chloride was measured with KC-4A. PAI-1 antigen levels were measured by sandwich ELISA using Assay Max® PAI-1 ELISA Kit (Assay Pro, MO). FDP concentrations were measured by a latex agglutination FDLP test (Kyowa Medex, Tokyo, Japan). TF procoagulant activities in plasma were measured by a one-stage clotting assay, as described previously [17-18]. TF procoagulant activities were quantitated by reference to standard curves (log-log plot) constructed with rabbit brain thromboplastin. One unit of activity was defined as a clotting time of 20 s in a standard assay with normal rat plasma. The measured coagulation activity was confirmed to be due to TF by negating the activity with anti-TF peptide E-76 [19].

Detection of histological change in the renal glomeruli in LPS-treated rat: The kidneys were excited and fixed in 10% neutral buffered formalin. Sections of the kidneys were histologically stained using phosphotungstate / hematoxylin (PTAH) to detect fibrin thrombi [20].

Statistical analysis: Data are expressed as the mean ± standard deviation (SD). Results were analyzed using Student's *t*-test, and statistical significance for all comparisons was assigned at $p < 0.05$ or $p < 0.01$.
The paired Student's t-test was used to determine significant differences in the occlusion time in mice. Differences between groups in other experiments were assessed using an analysis of variance.

RESULTS

Measurement of PLT count: Activated PLTs adhere to vascular endothelial cells, release coagulation factors, and are involved in thrombus formation. The decrease of PLT count reflects thrombus formation in circulatory system. The PLT count was markedly decreased by 80% of control 4 h after LPS infusion ($p < 0.01$ vs control) (Figure 2A). Nobiletin pre-treatment (50 mg/kg) attenuated the LPS-induced decrease of PLTs in rat blood ($p < 0.05$ vs LPS).

Fbg concentration: The decrease in Fbg occurs by converting from Fbg to fibrin by thrombin and clearly shows developing thrombus formation in rat. Fbg concentration in rat plasma decreased 69% against control level after LPS infusion, $p < 0.01$ vs control (Figure 2B). Nobiletin (50 mg/kg) attenuated the LPS-induced decrease of Fbg in rat blood ($p < 0.05$ vs LPS).

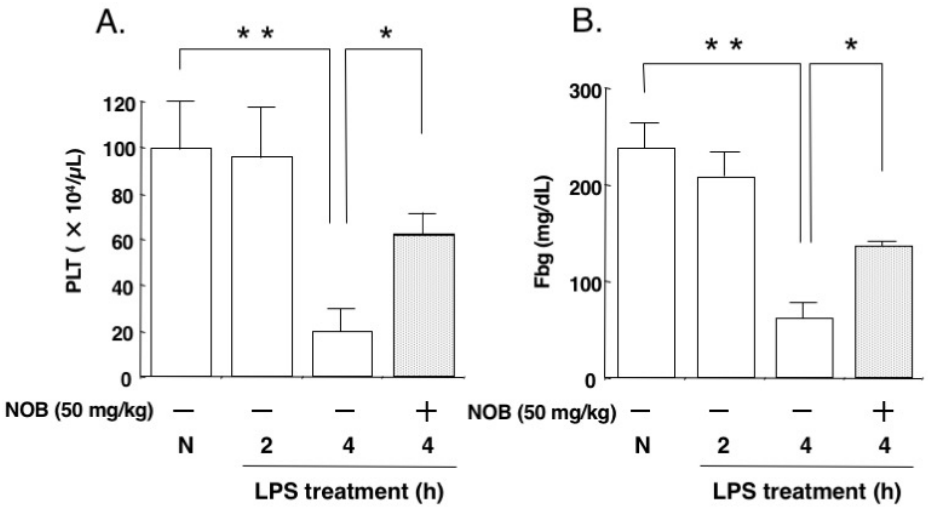


Figure 2. Nobiletin represses the decrease of PLT counts and Fbg in LPS-infused rats plasma. Rats were infused with LPS (12.5mg/kg/h) for 2 or 4h. Control rats were infused with saline for 4h. Blood samples were collected into tubes containing a 1/10 volume of 3.8% sodium citrate. Platelets in the blood (A) were counted with a Coulter Counter. The content of Fbg in plasma (B) were determined by a coagulation time assay. Experiments were conducted 3 animals per a group. Results are expressed as percentages of control value and represent the mean from four independent experiments (n = 4). Significantly different among each result was shown as * $p < 0.05$ or ** $p < 0.01$.

PT, APTT: The extension of PT or APTT is induced by the abnormality of the external or the internal coagulation system, respectively. Although both PT and APTT were prolonged by LPS treatment, the prolongation of APTT was greater than that of PT (Figure 3A, B), and the extent of prolongation of APTT was more remarkable (256%) than that of PT (180%) (Figure 3A, B). Nobiletin (50 mg/kg) ameliorated the LPS-induced prolongation of PT and APTT in rat blood. However, significant differences were not detected in either parameter (p = 0.07 for PT; p = 0.13 for APTT).

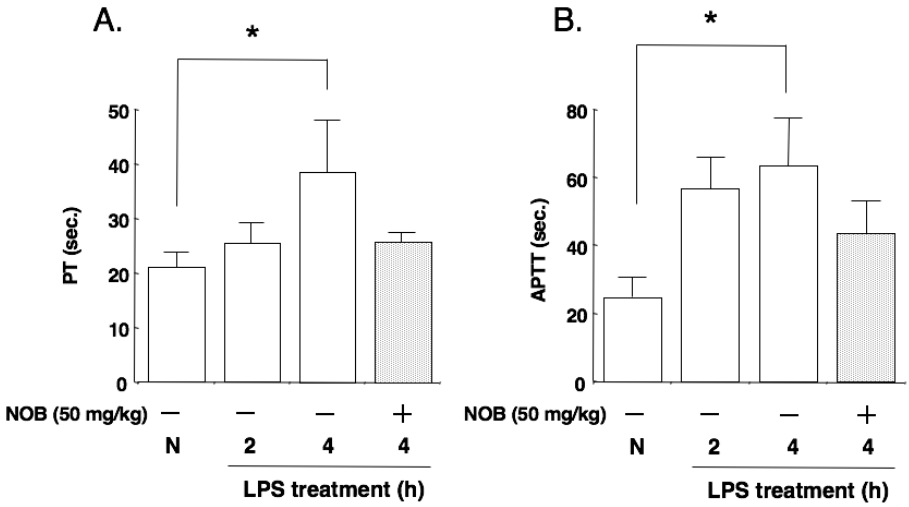


Figure 3. Nobiletin represses the extension of PT and APTT in LPS-infused rats plasma. Rats were infused with LPS (12.5mg/kg/h) for 2 or 4h. Control rats were infused with saline for 4h. Blood samples were collected into tubes containing a 1/10 volume of 3.8% sodium citrate. PT (A), and APTT (B) in plasma were determined by a coagulation time assay. Experiments were conducted 3 animals per a group. Experiments were conducted 3 animals per a group. Results are expressed as percentages of control value and represent the mean with standard deviation (SD) from four independent

experiments (n = 4). * indicates a significant difference ($p < 0.05$).

TF activity: TF is known as a trigger of blood coagulation cascade. TF activity was barely detected in normal rat plasma. It was markedly increased to 5,375% of the normal level after treatment with LPS for 4 h ($p < 0.05$). This result showed that the blood coagulation via the external coagulation system in rats was markedly advance in this time. Nobiletin suppressed the increase of TF activity in rat plasma 92% after 4 h (Figure 4A).

PAI-1 antigen levels: PAI-1 antigen levels in plasma were scarcely observed 2 h after LPS treatment but increased to 182% of normal level after 4 h ($p < 0.01$). The increase of PAI-1 induced stabilization of fibrin by the inhibition of plasmin. Nobiletin suppressed the increase of PAI-1 expression 83% at 4 h (Figure 4B).

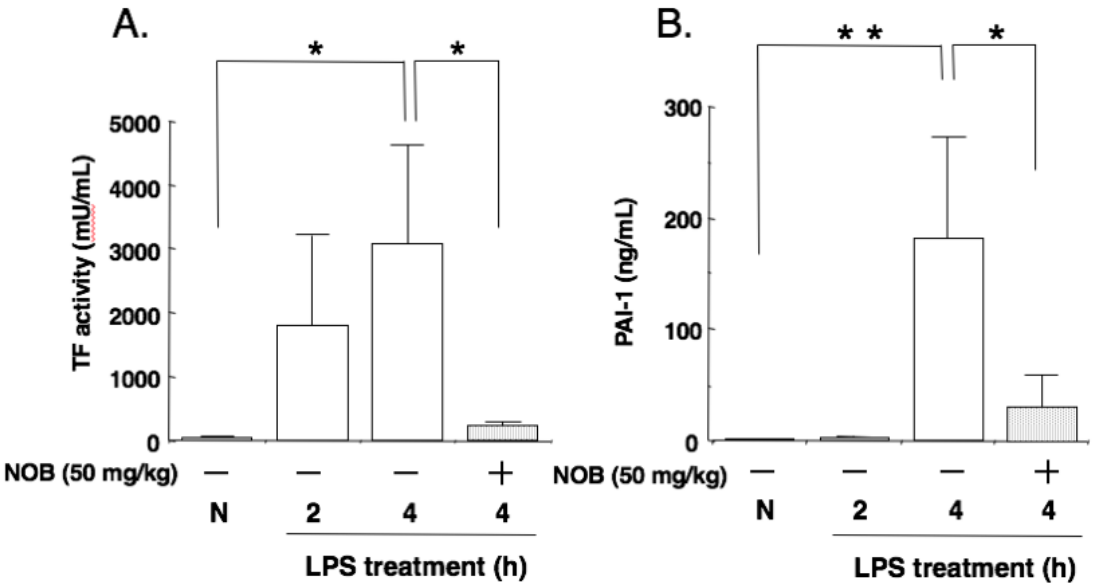


Figure 4. Nobiletin decreases TF activity and PAI-1 antigen levels in LPS-infused rats. Rats were infused with LPS (12.5mg/kg/h) for 2 or 4h. Control rats were infused with saline for 4h. Blood samples were collected into tubes containing a 1/10 volume of 3.8% sodium citrate. TF activity (A) was measured by a one-stage clotting assay. Experiments were conducted using 3 animals per a group. PAI-1 antigen level in rat plasma (B) were determined by ELISA. Results are expressed as percentages of control value and represent the mean with standard deviation (SD) from four independent experiments (n = 4). * indicates a significant difference ($p < 0.05$) or ** $p < 0.01$.

FDP concentration: FDP is known as a typical marker of fibrinolysis system activation. An increase in the FDP concentration was observed 4 h after LPS infusion, but it remained in the normal range (under 10 μg/mL)(data not shown).

Histological study of microthrombi in kidneys of LPS-treated rats: Histological staining for PTAH showed that infusion of LPS caused formation of distinct thrombi in the renal glomeruli. In PTAH staining nuclei and fibrin are dyed blue. The fibrin thrombi were recognized in glomerular capillaries of LPS treated rat (Figure 5B) as compared to control (Figure 5A). Nobiletin markedly repressed fibrin deposition in the renal glomeruli induced by LPS treatment (Figure 5C).

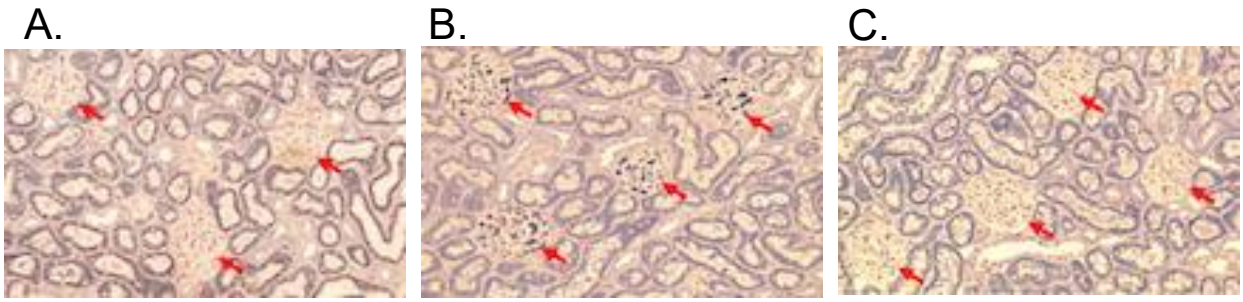


Figure 5. Nobiletin repressed microthrombi formation in LPS-infused rat kidney. Rats were infused with LPS (12.5mg/kg/h) for 4h. Control rats were infused with saline for 4h. Rats were sacrificed, and the kidneys were excited and fixed in 10% neutral buffered formalin. Sections of the kidneys were histologically stained using phosphotungstate / hematoxylin (PTAH) to detect fibrin thrombi using optical microscope (200 x). Pictures show the condition of rat renal glomeruli after different treatment. Control (A), LPS (B), nobiletin + LPS (C), respectively. The renal glomeruli in each picture were indicated by red arrows.

DISCUSSION

We examined the inhibitory effects of nobiletin on DIC by using an LPS-induced rat model of DIC. Departure from normal levels was observed after 4 h for all examined blood coagulation parameters after LPS administration. However, the amount of FDP (fibrinolytic activation) did not increase significantly after LPS treatment at 4 h. This result confirmed that this model is a DIC model with weak fibrinolysis as previously reported [5]. In nobiletin-administered rats, LPS-mediated deviations from the normal levels were suppressed or alleviated.

TF activity sharply increased by about 54-fold after LPS treatment, but nobiletin suppressed most of the increase (92%). In particular, the plasma PAI-1 antigen level was increased about 86-fold by LPS, but nobiletin inhibited the increase by 83%.

The suppressive effects of nobiletin against LPS-mediated indices such as TF and PAI-1 were higher than the effects against coagulation reactions such as PLT count, PT, APPT and Fbg. These findings suggest that nobiletin suppresses the activation of coagulation factors or reflects the suppression of the transcriptional activation of a protein that triggers coagulation rather than inhibiting the activity of coagulation factors once activated.

In previous study, we determined that TF was expressed in leukocytes and vascular endothelial cells through the activation of transcriptional factors following 2 to 4 h exposure to LPS *in vitro* [13]. PAI-1 antigen is expressed by leukocytes [21], endothelial cells [22], liver [23] and adipocytes [23] in a similar fashion. On the other hand, PAI-1 is present in vascular endothelial cells [24] and platelets [25] and is released to the blood following vascular endothelial damage and PLT disruption [25]. From our results, it appears that the increase in blood PAI-1 antigen is induced by release from PLTs and activation of transcription in other cells.

The presented results seemed to reflect the inhibition mechanisms that were shown in our previous *in vitro* studies. The original experiments showed that nobiletin inhibits transcription factors such as NF□B, AP-1 and Sp1 *in vitro* [13]. The current experiments suggest that nobiletin also suppresses the expression of those proteins as *in vivo*. Nobiletin was thought to suppress the expression of TF and PAI-1 by efficiently suppressing these transcriptional pathways (Figure 4). Therefore, it seemed that the LPS-induced DIC model used here was

an appropriate model for investigating the effects of nobiletin. However, it is not clear which signals have been inhibited in the transcriptional activation pathway by nobiletin, and additional research is required to answer that question. The thromboplastin-induced DIC model may not show pronounced inhibitory effects of nobiletin because it is directly administered intravascularly and blood coagulation reaction proceeds promptly.

We hypothesize that lifestyle-related diseases involving thrombus formation could be reduced by inclusion of nobiletin in the diet. Nobiletin is extracted from shiikuwasa (*Citrus depressa* Hayata, Rutaceae), a popular citrus fruit in Okinawa, Japan. Since nobiletin and other flavonoids are abundantly contained in the peel of citrus fruit [26-27], it is likely that greater anti-thrombotic effects could be achieved if the juice containing peel were consumed together. The amount of nobiletin used in this study was 50 mg/kg single dose. In the case of humans at weight 50 kg, consuming this does equates to drinking with fruit juice made from 6 or 7 fruits of shiikuwasa with a diameter of 1.2 inch. No side effects or pathological changes were observed, even following continuous administration for 7 days in a separate experiment in rat (data not shown).

In addition to traditional antioxidant activity, various biological activities are attracting attention to flavonoid compounds [28-29] and applications are expected. In nobiletin, the 5, 6, 7, 8, 3', 4' positions are methoxy groups, making it more lipophilic than if they were hydroxyl groups. It is currently unknown how these characteristics affect the results presented here. It is known that nobiletin is metabolized to 3'-OH, 4'-OH, or 3', 4'-OH forms and released into the urine [30-32]. The anti-inflammatory effects of these metabolites are drawing attention. In recent years, it has been reported that metabolites of nobiletin inhibit inflammation [33]. Important studies examining the relationship between structure and activity should be conducted. Such research may lead to the development of new anticoagulants of familiar food origin and the maintenance of people's health in the future.

CONCLUSION

Ingestion of nobiletin reduced LPS-induced DIC symptoms. At this time, the fluctuations of TF and PAI-1 were more strongly suppressed than the fluctuations of blood indices related to the coagulation reaction such as PLT count, PT, APTT, and Fbg. This is because the inhibitory effect of nobiletin on the intracellular signal transduction by LPS is stronger than the inhibitory effect on the activation of various proteases involved in the blood coagulation cascade.

Based on the above, it was considered that nobiletin suppresses blood coagulation and maintains normal blood flow through suppressing activation of the transcription pathway of the protein that causes blood coagulation, and as a result LPS-induced DIC symptoms are alleviated.

List of Abbreviations: DIC: disseminated intravascular coagulation, LPS: lipopolysaccharide, PLT: platelet, PT: prothrombin time, APTT: activated partial thromboplastin time, Fbg: fibrinogen, TF: tissue factor, PAI-1: plasminogen activator inhibitor-1, TAFIL: thrombin-activatable fibrinolysis inhibitor, FDP: fibrin degradation products, PRP: platelet-rich plasma, PPP: platelet-poor plasma.

Competing Interests: There are no conflicts of interest to declare.

Author's Contribution: Kimihiko Takada designed, analyzed data and contributed fundamental conceptualization for the research. Mayuko Takano, Aiko Kunii and Kei Harayama performed the experiment

in part of analysis of the coagulation indicators. Yoshiko Miyazaki and Yutaka Masuda critically revised the manuscript. All authors read and approved the final version of the manuscript.

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REFERENCES

1. Mammen EF: Disseminated intravascular coagulation (DIC). Clin. Lab. Sci. 2000, 13 (4), 239-245.
2. Bakhtiari K, Meijers JC, de Jonge E, Levi M: Prospective validation of the international society of thrombosis and haemostasis scoring system for disseminated intravascular coagulation. Crit. Care Med. 2004, 32 (12), 2416–2421.
3. Zeerleder S. Hack CE. Wuillemin WA: Disseminated intravascular coagulation in sepsis. Chest 2005, 128 (4), 2864–2875.
4. Garner R. Evensen SA. Endotoxin-induced intravascular coagulation and shock in dogs: the role of factor VII. Br J Haematol. 1974, 27 (4), 655-668.
5. Asakura H. Suga Y. Yoshida T. Ontachi Y. Mizutani T. Kato M. Ito T. Morishita E. Yamazaki M. Miyamoto K. Nakao S: Pathophysiology of disseminated intravascular coagulation (DIC) progresses at a different rate in tissue factor-induced and lipopolysaccharide-induced DIC models in rats. Blood Coagul. Fibrinolysis. 2003, 14 (3), 221-228.
6. Asakura H. Okudaira M. Yoshida T. Ontachi Y. Yamazaki M. Morishita E. Miyamoto K. Nakao, S: Induction of vasoactive substances differs in LPS-induced and TF-induced DIC models in rats. Thromb. Haemost. 2002, 88 (4), 663-667.
7. Krishnamurti C. Young GD. Barr CF. Colleton CA. Alving BM: Enhancement of tissue plasminogen activator-induced fibrinolysis by activated protein C in endotoxin-treated rabbits. J. Lab. Clin. Med. 1991, 118 (6), 523-530.
8. Zeuke S. Ulmer AJ. Kusumoto S. Katus HA. Heine H: TLR4-mediated inflammatory activation of human coronary artery endothelial cells by LPS. Cardiovasc. Res. 2002, 56 (1), 126-134.
9. Rumbaut RE. Bellera RV. Randhawa JK. Shrimpton CN. Dasgupta SK. Dong JF. Burns AR: Endotoxin enhances microvascular thrombosis in mouse cremaster venules via a TLR4-dependent, neutrophil-independent mechanism. Am. J. Physiol. Heart. Circ. Physiol. 2006, 290 (4), H1671-H1679.
10. Lin N. Sato T. Takayama Y. Mimaki Y. Sashida Y. Yano M. Ito A: Novel anti-inflammatory actions of nobiletin, a citrus polymethoxy flavonoid, on human synovial fibroblasts and mouse macrophages. Biochem. Pharmacol. 2003, 65 (12), 2065-2071.
11. Kawabata K. Murakami A. Ohigashi H: Nobiletin, a citrus flavonoid, down-regulates matrix metalloproteinase-7 (matrilysin) expression in HT-29 human colorectal cancer cells. Biosci. Biotechnol. Biochem. 2005, 69 (2), 307-314.
12. Sato T. Koike L. Miyata Y. Hirata M. Mimaki Y. Sashida Y. Yano M. Ito, A: Inhibition of activator protein-1 binding activity and phosphatidylinositol 3-kinase pathway by nobiletin, a polymethoxy flavonoid, results in augmentation of tissue inhibitor of metalloproteinases-1 production and suppression of production of matrix metalloproteinases-1 and -9 in human fibrosarcoma HT-1080 cells. Cancer Res. 2002, 62 (4), 1025-1029.
13. Hirata Y. Masuda Y. Kakutani H. Higuchi T. Takada K. Ito A. Nakagawa Y. Ishii H:Sp1 is an essential transcription factor for LPS-induced tissue factor expression in THP-1 monocytic cells, and nobiletin represses the expression through inhibition of NF-?B, AP-1, and Sp1 activation. Biochem. Pharmacol. 2008, 75 (7), 1504-1514.
14. Takada K. Seike T. Sasaki T. Masuda Y. Ito A. Ishii H: Nobiletin, a polymethoxyflavone in citrus fruits, reduces TAFI expression in HepG2 cells through transcriptional inhibition. Thromb. Haemost. 2013, 109 (6), 1060-1069.

15. Lu WJ. Lin KC. Liu CP. Lin CY. Wu HC. Chou DS. Geraldine P. Huang SY. Hsieh CY. Sheu JR: Prevention of arterial thrombosis by nobiletin: in vitro nad in vivo studies. J. Nutr. Biochem. 2016, 28, 1-8.
16. Ishiwa J. Sato T. Mimaki Y. Sashida Y. Yano M. Ito A: A citrus flavonoid, nobiletin, suppresses the production and gene expression of matrix metalloproteinase 9 / gelatinase B in rabbit synovial fibroblasts. J. Rheumatol. 2000, 27 (1), 20–25.
17. Ishii H. Horie S. Kizaki K. Kazama M: Retinoic acid counteracts both the downregulation of thrombomodulin and the induction of tissue factor in cultured human endothelial cells exposed to tumor necrosis factor. Blood 1992, 80 (10), 2556-2562.
18. Ishii H. Takada K. Higuchi T. Sugiyama J: Verotoxin-1 induces tissue factor expression in human umbilical vein endothelial cells through activation of NF□B/Rel and AP-1. Thromb. Haemostat. 2000, 84 (4), 712-721.
19. Dennis MS. Eigenbrot C. Skelton NJ. Ultsch MH. Santell L. Dwyer MA. O'Connell MP. Lazarus RA: Peptide exosite inhibitors of factor VIIa as anticoagulants. Nature 2000, 404 (6777), 465–470.
20. Muto Y. Suzuki K. Sato E. Ishii H: Carboxypeptidase B inhibitors reduce tissue factor induced renal microthrombi in rats. Eur. J. Pharmacol. 2003, 461 (2-3), 181-189.
21. Hamilton JA. Whitty GA. Wojta J. Gallichio M. McGrath K. Ianches G: Regulation of plasminogen activator inhibitor-1 levels in human monocytes. Cell. Immunol. 1993, 152 (1), 7- 17.
22. Scheleef RR. Loskoutoff DJ: Fibrinolytic system of vascular endothelial cells. Role of plasminogen activator inhibitors. Haemostasis 1988, 18 (4-6), 328-341.
23. Sawdey MS. Loskutoff DJ: Regulation of murine type 1 plasminogen activator inhibitor gene expression in vivo. J. Clin. Invest. 1991, 88 (4), 1346-1353.
24. Huebner BR. Moore EE. Moore HB. Gonzalez E. Kelher MR. Sauaia A. Banerjee Silliman CC: Thrombin stimulates increased plasminogen activator inhibitor-1 release from liver compared to lung endothelium. J. Surg. Res. 2018, 225, 1-5.
25. Nylander M. Osman A. Ramström S. Aklint E. Larsson A. Lindahl TL: The role of thrombin receptors PAR1 and PAR4 for PAI-1 storage, synthesis and secretion by human platelets. Thromb. Res. 2012, 129 (4), e51-58.
26. Horowitz RM. Gentilli B: Flavonoid constituents of citrus. In Citrus Science and Technology, Nagy S. Shaw PE. Veldhuis MK.(eds). Avi, Westport, CT, 1977; 397.
27. Li S. Yu H. Ho CT: Nobiletin: efficient and large quantity isolation from orange peel extract. Biomed. Chromatogr. 2006, 20 (1), 133-138.
28. Montanari A. Chen J. Widmer W: Citrus flavonoids: a review of past biological activity against disease. Discovery of new flavonoids from Dancy tangerine cold pressed peel oil solids and leaves. Adv. Exp. Med. Biol. 1998, 439, 103-116.
29. Wang M. Meng D. Zhang P. Wang X. Du G. Brennan C. Li S. Ho CT. Zhao H: Antioxidant protection of nobiletin, 5-Demethylnobiletin, Tangeretin, and 5-Demethyltangeretin from citrus peel in saccharomyces cerevisiae. J. Agric. Food Chem. 2018, *66* (12), 3155-3160.
30. Li S. Wang Z. Sang S. Huang MT. Ho CT: Identification of nobiletin metabolites in mouse urine. Mol. Nutr. Food Res. 2006, 50 (3), 291-299.
31. Li S. Sang S. Pan MH. Lai CS. Lo CY. Yang CS. Ho CT: Anti-inflammatory property of the urinary metabolites of nobiletin in mouse. Bioorg. Med. Chem. Lett. 2007, 17 (18), 5177-5181.
32. Lai CS. Li S. Chai CY. Lo CY. Dushenkov S. Ho CT. Wang YJ: Anti-inflammatory and antitumor promotional effects of a novel urinary metabolite, 3′,4′-didemethylnobiletin, derived from nobiletin. Carcinogenesis. 2008, 29 (12) 2415-2424.
33. Wu X. Song M. Rakariyatham K. Zheng J. Guo S. Tang Z. Zhou S. Xiao H: Anti-inflammatory effects of 4′-demethylnobiletin, a major metabolite of nobiletin. J. Funct. Foods. 2015, 19 (Pt A), 278-287